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(54) Title: PROTEASE-DEFICIENT GRAM-POSITIVE BACTERIA AND THEIR USE AS HOST ORGANISMS FOR THE PRODUCTION OF RECOMBINANT PRODUCTS

(57) Abstract

The residual protease activity in AP-/NP- strains of *Bacillus* is due to two additional proteases, a residual serine protease (RSP) and a sulfhydryl-dependent residual cysteine protease (RCP), which together account for the degradation of recombinant proteins in cultures of *B. subtilis* (AP-/NP-). An analysis has been developed which allows for the identification and development of mutant strains of *B. subtilis* which are deficient in not only the neutral and alkaline proteases, but also in one or both of the residual proteases. These strains are highly suitable for use as host organisms for the production of heterologous proteins which are susceptible to the activity of the residual proteases.

Amino Terminal Sequence of RSP

Leu Ile Gly Lys Glu Val Leu Pro Phe Glu Ala Lys Ala Phe Lys Asn
1 2

Gly Glu Phe Ile Asp Val Thr Asn Glu Asp Leu Lys Gly Gln Trp Ser
3

Ser Val Phe Phe Phe Tyr Pro Ala

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Description

Protease-Deficient Gram-Positive Bacteria and Their Use as Host Organisms for the Production of Recombinant Products

This invention relates to strains of gram-positive bacteria such as Bacillus subtilis which are protease-deficient to methods and tools useful in the isolation of such strains and to the use of these strains for the production of heterologous proteins.

Background of the Invention

The successful introduction of foreign genes into bacteria, and their expression for the production of medically and industrially important proteins by recombinant DNA technology is one of the most significant technological advances of the last decade.

The most widely used host organism for the expression of cloned genes is E. coli. Heterologous proteins produced in E. coli, however, accumulate in the cytoplasm of the cell. Frequently, the heterologous product accumulates as an insoluble product in inclusion bodies. Thus, the production of heterologous proteins in E. coli and other gram-negative hosts requires extensive purification of the desired product from contaminating host proteins. Moreover, excessively harsh, denaturing conditions may be required to extract the protein from inclusion bodies leading to significant inactivation of the protein and thus poor recovery. Additional problems can result when the recombinant protein contains sulfhydryl groups. Frequently, random polymerization of proteins through nonspecific disulfide crosslinking occurs during extraction.

Bacillus and other gram-positive organisms will, on the other hand, secrete proteins into the extracellular culture medium. This is advantageous

from the standpoint of purification of heterologous proteins. Moreover, bacteria such as Bacillus are more cost effective in production of proteins than competing hosts, such as yeast and mammalian cell cultures due to higher growth rates, the use of less expensive growth media, and generally more facile cultivation procedures.

Bacillus is a fermentation organism grown routinely on an industrial scale, and one species, B. subtilis, is the most widely studied gram-positive organism. It is non-pathogenic, and unlike E. coli, it does not produce endotoxin, clearly desirable features when the recombinant products are destined for medical or veterinary use. Plasmid cloning vectors have been constructed which make it possible to introduce and express foreign genes in Bacillus hosts. In general, these cloning vectors are secretion vectors, in which the signal sequence of a secretory Bacillus protein is fused to the structural gene coding for the desired heterologous protein. This construct is then cloned into a plasmid capable of replicating in Bacillus. These plasmids have unique restriction sites, and encode selectable antibiotic resistance markers useful in isolation of transformant organisms.

Despite the great potential for gram-positive bacteria such as B. subtilis as hosts, the general utility of these organisms for the production of heterologous proteins has remained limited for a number of reasons, not the least being the occurrence of protein degradation. This problem is significant because Bacillus produces at least three reported extracellular proteases which can attack and degrade heterologous proteins produced by the Bacillus. Priest, 41 Bacteriol. Rev. 711 (1977); Roitsch et al., 155 J. Bacteriol. 145 (1983); Stahl et al., 158 J. Bacteriol. 411 (1984); Uehara et al., 139

J. Bacteriol. 583 (1979); Yang et al., 160 J. Bacteriol. 15 (1984).

The two major Bacillus extracellular proteas s are the neutral protease (NP), a metalloenzyme sensitive to ethylenediaminetetraacetic acid (EDTA), and the alkaline protease (AP), a serine protease having optimum activity at alkaline pH. The structural genes for these two major proteases have been cloned and used to produce in vitro derived defined deletions which inactivate the respective protease genes. Stahl et al., 158 J. Bacteriol. 411 (1984); Yang et al., 160 J. Bacteriol. 15 (1984); Kawamura et al., 160 J. Bacteriol. 442 (1984). These mutant strains with deletions in the structural genes for the two major proteases are referred to herein as $aprE^-$ /$nprE^-$ double mutants. Mutant strains which are phenotypically deficient in alkaline and neutral protease, but in which the nature of the mutation is not specified are referred to herein as AP^-/NP^- double mutants.

The proteolytic activities of wild type B. subtilis strains, such as BD170 (ATCC 33608), and AP^-, NP^-, and AP^-/NP^- mutants have been evaluated by growing the strains on casein- agar and observing the size of clear halos formed surrounding colonies which produce proteases capable of degrading casein. Figure 1 shows growth of wild-type and protease-deficient mutants of B. subtilis BD170 on TBAB agar containing 1% skim milk at 37°C for 16h are shown. Clear zones surrounding the colonies are due to proteolytic degradation of casein. Strains are (1) wild-type, (2) alkaline protease minus (AP^-), (3) neutral protease minus (NP^-), and (4) double protease minus (AP^-/NP^-). The protease-deficient double mutants (AP^-/NP^-) exhibit little or no activity toward casein in this assay as shown in Fig. 1. Nevertheless, AP^- /NP^- strains of B. subtilis still degrade many recombinant secretory proteins of interest. For example,

AP^-/NP^- strains of B. subtilis degrade heterologous proteins such as lysostaphin, prolysostaphin, micrococcal nuclease, and fusion proteins comprising eukaryotic proteins, including interleukin-1 and tissue-plasminogen activator, fused with the secretory and proenzyme sequences of prolysostaphin to enable their secretion from the bacterium.

It is an object of this invention to provide a method for screening and identifying gram-positive organisms, particularly of the genus Bacillus which possess a substantial reduction in residual protease activity.

It is an object of this invention to provide gram-positive host organisms, particularly of the genus Bacillus, which are superior to currently available protease-negative double mutants because of a substantial reduction in the residual protease activity.

It is a further object of this invention to provide a method of producing highly protease-deficient strains of gram-positive bacteria, especially Bacillus spp., for use as host organisms in the production of heterologous proteins.

It is another object of the invention to provide an improved method for producing heterologous proteins which makes use of the highly protease-deficient gram-positive bacteria as hosts

Summary of the Invention

It has now been found that the residual protease activity in AP^-/NP^- strains of Bacillus is due to two additional proteases, a residual serine protease (RSP) and a sulfhydryl-dependent residual cysteine protease (RCP), which together account for the degradation of recombinant heterologous proteins in cultures of B. subtilis (AP^-/NP^-). The activity of these two enzymes was investigated and it was found that the two proteases possess different activities towards

heterologous proteins, for example, lysostaphin and prolysostaphin. Further, the N-terminal amino acid sequence of RSP was determined and nucleic acid probes were designed for use in site specific mutation of the RSP gene. Using these tools, strains of B. subtilis can be developed and identified which are deficient in not only the neutral and alkaline proteases, but also in one or both of the residual proteases. Such strains are highly suitable for use as host organisms for the production of heterologous proteins which would be susceptible to the proteolytic activity of the residual proteases.

Brief Description of the Drawings

Fig. 1 shows a casein-agar plate demonstrating the protease activity of wild type B. subtilis BD170, and its AP^-/NP^- and AP^-/NP^- mutants;

Fig. 2 shows the halflife of a recombinant protein, lysostaphin, throughout growth in cultures of B. subtilis BD170 wild type, B. subtilis BD170 AP^-/NP^-, and the protease-deficient isolate B. sphaericus 00;

Fig. 3 shows the chromatographic separation of the residual cysteine protease (RCP) and the residual serine protease (RSP);

Fig. 4 shows the respective activities of RCP and RSP toward specific polypeptide substrates, prolysostaphin and lysostaphin;

Fig. 5 shows the effect of inhibition of RCP and RSP on the stability of a heterologous secretory protein;

Fig. 6 shows the N-terminal amino acid sequence of RSP; and

Fig. 7 shows the preferred oligonucleotide probe sequence for RSP.

Detailed Description of the Invention

In order to establish the cause of the residual protease activity in AP^-/NP^- strains of Bacillus, culture supernatant from B. subtilis BD170 AP^-/NP^- was evaluated. Two proteins, a residual serine protease (RSP) and a sulfhydryl-dependent residual cysteine protease (RCP) were identified which accounted for all of the residual extracellular protease activity. These two proteins were isolated from late stationary phase culture supernatant as described below. Following isolation, RSP was found to be inactivated by phenylmethylsulfonyl fluoride (PMSF) and inhibited by the enzyme inhibitor antipain (Phe-CO-Arg-Val-Arg-al). RCP was found to be inactivated by p-hydroxymercuribenzoate (pHMB) and (inhibited) by antipain, indicating that RSP and RCP are distinct proteins.

Figure 2 shows lysostaphin inactivation as a function of growth stage comparing B. subtilis BD170 wild-type (W), with B. subtilis AP^-/NP^- (D), and B. sphaericus 00 protease-deficient (S). Supernatant samples were collected at the end of logarithmic growth and throughout stationary phase. Supernatant (20ul) was incubated with 20 ul of 1.0 mg/ml lysostaphin solution for 30 min at 37°C, after which the samples were rapidly frozen on dry-ice to arrest further inactivation. Residual lysostaphin activity was then measured turbidometrically toward heat killed Staphyloccus aureus and compared with control lysostaphin.

The halflife of recombinant lysostaphin (e.g. lysostaphin produced by recombinant DNA technology as described in International Patent Application Serial No. PCT/US87/00873 published as WO87/06264 on October 22, 1987 and incorporated herein by reference) is similar in the stationary phase culture supernatants of both wild type B. subtilis and its AP^-/NP^- mutants. This key observation illustrates that the

instability of this secretory recombinant product is largely effected by the activity of the residual proteases, residual cysteine protease (RCP) and residual serine protease (RSP) in those cultures. Moreover, this result also indicates that the generally held concept that the major extracellular protease activity in wild type B. subtilis culture supernatants is due to the neutral and alkaline proteases is in fact misleading.

The activity of RSP and RCP in degrading a heterologous protein such as lysostaphin was further investigated as a possible basis for an assay technique for identifying protease deficient Bacillus strains. Figure 4 shows analysis for RCP and RSP activities after fractionation of B. subtilis BD170 AP^-/NP^- stationary phase culture supernatant by chromatography on Mono Q. Samples (20 ul) of column fractions (1.0 ml) were incubated with 20 ul of either (A) prolysostaphin the presence of 1.0 mM phenylmethylsulfonyl fluoride (PMSF), or (B) lysostaphin for 30 minutes at 25°C, and then rapidly frozen on dry-ice to arrest further degradation. The samples were then analyzed by SDS-PAGE and stained with Coomassie Blue. The chromatographic conditions were by anion exchange HPLC on a column of Mono Q (Pharmacia) using the following conditions: Solvent A: water, Solvent B: 100 mM Na_2HPO_4, pH 7.0, 1.0M in NaCl. Gradient: 5-100% solvent B in 15 minutes at 1.0 ml per minute. Peaks were detected on-line by A_{210} and A_{280}. The positions of the arrows indicate the beginning and end of the salt gradient.

RSP can degrade both of the heterologous proteins prolysostaphin and lysostaphin (Fig. 4). On the other hand, RCP has no effect on lysostaphin, but can convert prolysostaphin to lysostaphin. Prolysostaphin is inactive and the conversion of the heterologous protein prolysostaphin to enzymically active lysostaphin forms the basis for an assay for RCP. The

inactivation that accompanies the degradation of the heterologous protein lysostaphin forms the basis of an assay for RSP.

The knowledge concerning the two residual proteases and their reaction with heterologous protein permits naturally occurring Bacillus strains to be screened to locate those strains that lack these protease activities. In this way, an $AP^-/NP^-/RSP^-/RCP^-$ strain of B. sphaericus, B. sphaericus 00, has been identified. A substantially pure culture of this host strain transformed with a plasmid carrying the gene for lysostaphin has been deposited with the American Type Culture Collection and assigned accession No. 67080 in connection with U.S. Patent Application Serial No. 034,464 corresponding to PCT/US87/00873. The unmodified host is maintained in the culture collection of the Public Health Research Institute, New York, New York. This host is of importance not only because it is $AP^-/NP^-/RSP^-/RCP^-$ but in that it can be transformed by plasmids pBC16-1L and pROJ6499-1L to produce and process prolysostaphin to lysostaphin despite the absence of AP, NP, RSP, and RCP protease activities.

Further, with the knowledge that there are two residual proteases in B. subtilis AP^-/NP^- strains and assay systems based on their reaction with heterologous proteins capable of distinguishing their activities, mutant strains deficient in either or both of the residual proteases can be selected from mutant populations created by any known method. Mutant strains are strains of microorganisms having one or more mutations, i.e. changes, in the genetic sequence of the organism. These mutations can be caused by any of a variety of mutagenic methods including exposure to radiation or chemical mutagens, transposition, recombination and restriction enzyme digestion. The resulting mutant strain can differ from the parent strain due to base-pair replacement, i.e. the

substitution of one base-pair for another in the genetic sequence of the organism (base-pair replacement mutants); the insertion of one or more base-pairs into the genetic sequence (insertion mutants); or the deletion of one or more base pairs from the genetic sequence (deletion mutants). In practice, deletion mutants are preferred because of the possibility of reversion in base-pair replacement or insertion mutants. Such deletion mutants are preferably formed by known methods for specific in vitro deletions in the appropriate gene.

Knowing the identity of the residual protease, it is also possible to eliminate the effect of the residual RCP and RSP produced by a non-mutant microorganism by chemically inhibiting or inactivating the effect of the RCP and RSP as they are produced in the culture medium by addition of chemicals for example pHMB, PMSF, and peptide analogue inhibitors such as antipain and metal chelators such as EDTA.

The first step in creating in vitro site specific deletion mutants deficient in RSP and/or RCP activity requires that the respective genes coding for these proteases within the bacterial genome be cloned.

Toward this end, the N-terminal amino acid sequence of RSP has been determined, as shown in Fig. 6. Based on the amino acid sequence of the protein, regions of least ambiguous DNA sequence, as predicted from codon degeneracy, were identified. Oligonucleotide probes corresponding to these amino acid sequences can be synthesized for use in hybridization studies to identify restriction fragments containing the residual protease gene. As appropriate, the codon degeneracy is accommodated by the use of mixed base oligonucleotide probes. For example, three such oligonucleotide mixed probes appropriate for regions of the N-terminal amino acid sequence generated for RSP are shown in Fig. 7. Other

probe sequences based on other parts of the protease amino acid sequence can also be used.

Once suitable probe sequences have been identified, the genes encoding RSP and RCP can be located by hybridizing the ^{32}P-labeled oligonucleotide probes with restriction digests of Bacillus chromosomal DNA. The specific fragments which are identified as containing the RSP or RCP gene then are cloned in a plasmid and can be sequenced or subjected to site specific mutagenesis preferably to create deletion mutants. To facilitate selection of protease-deficient deletion mutants, antibiotic resistance markers can be inserted into the deleted gene. The protease genes in B. subtilis are then inactivated by homologous recombination and mutants are selected by growth in the presence of antibiotic and tested for residual protease activity.

The person skilled in the art will appreciate that given the heterologous protein based assay for residual protease activity and the amino acid sequence of RSP, that any number of residual protease-deficient mutants of Bacillus can be obtained or screened for. The procedures by which the assay for RSP and RCP is conducted, by which the amino acid sequence was determined, and by which specific mutants can be prepared are described in greater detail below.

Isolation and Characterization of RSP and RCP

To isolate and characterize the source of the residual protease activity of B. subtilis strain BD170 AP^-/NP^-, stationary phase culture supernatants were fractionated by ion-exchange high performance liquid chromatography (HPLC) on Mono Q (Pharmacia). Chromatography of B. subtilis BD170 AP^-/NP^- stationary phase culture supernatant, (B) rechromatography of RCP, and (C) rechromatography of RSP were done by anion exchange HPLC on a column of Mono Q (Pharmacia) using the following conditions: Solvent A: water, Solvent B: 100 mM Na_2HPO_4, pH 7.0, 1.0M in NaCl.

Gradient: 5-100% solvent B in 15 minutes at 1.0 ml per minute. Peaks were detected on-line by A_{210} and A_{280}. Two proteins, which together accounted for all the residual protease activity, were identified as a sulfhydryl-dependent protease (residual cysteine protease - RCP) and a serine protease (residual serine protease - RSP). These two proteins were isolated from stationary phase culture supernatant by ammonium sulfate precipitation (50% saturation) followed by ion-exchange HPLC on Mono Q using a linear salt gradient from 0.05 - 1.0 M NaCl in 0.05 M sodium phosphate buffer, pH 7.00. On rechromatography on Mono Q under identical conditions, RSP elutes as a symmetrical peak. RSP, so purified, yields a single band on SDS-PAGE with an apparent molecular weight near 18,000 daltons. RCP elutes from Mono Q early in the salt gradient as a heterogeneous peak. Column effluents were monitored simultaneously by on-line A_{210} and A_{280}.

Assays for RSP and RCP Using the Heterologous Protein Lysostaphin

The inactivation of lysostaphin that occurs in 30 minutes at room temperature when 20 µl of a 1.0 mg/ml solution of lysostaphin is incubated with an equal volume of culture supernatant (or other sample containing the RSP enzyme) forms the basis for the quantitative assay although other suitable heterologous proteins could be used as well. Shorter or longer time periods could be used, so long as the reaction is allowed to proceed for sufficient time to allow degradation of at least a part of the lysostaphin or other heterologous proteins if RSP is present. One unit of protease activity in this assay is the amount of protease that produces 50% inactivation of the lysostaphin incubated as described. The residual activity of the lysostaphin was measured turbidometrically by its activity against a cell suspension of heat killed Staphylococcus aureus and was compared with an untreated lysostaphin control.

Likewise, RCP can be quantitatively estimated under similar conditions with 1 unit being defined as that amount of RCP bringing about the 50% activation of prolysostaphin to lysostaphin. Since RSP will degrade lysostaphin produced from prolysostaphin by RCP, however, the assay for RCP in culture supernatants that also contain RSP requires that RSP be first inactivated by incubation with phenylmethysulfonyl fluoride (PMSF) or some other RSP inhibitor. In the presence of PMSF, the RCP activity of B. subtilis AP^-/NP^- cultures will convert prolysostaphin to lysostaphin and permit accumulation of the enzyme.

Alternatively, a convenient assay for RCP and RSP can be performed by analysis of heterologous proteins such as prolysostaphin and lysostaphin degradation and accumulation of degradation products by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4) Furthermore, the activity of RCP and RSP, respectively, can be quantitated by their activities towards heterologous proteins such as prolysostaphin and lysostaphin substrates labeled radioactively with ^{125}I or with fluorescein isothiocyanate. The degradation of the labeled heterologous proteins, or accumulation of degradation products, can be conveniently measured after acid precipitation to separate labeled products from labeled substrate and the measurement of either acid-soluble label or acid-insoluble label, respectively.

By using the above assays specific for residual protease activity, RSP was found to be inactivated by PMSF. Also, RSP is inhibited by the protease inhibitor antipain (Phe-Arg-Val-Arg-Al). RCP is unaffected by PMSF, but is inactivated by the sulfhydryl reactive agent, p-hydroxymercuribenzoate (pHMB). RCP is also inhibited by antipain.

With this knowledge, the residual protease activity from RSP and RCP can be specifically inactivated or inhibited so as to permit the accumulation of

heterologous proteins by cultures of Bacillus AP^-/NP^- strains (see Fig. 5). For example, figure 5 shows the production of interleukin 1-micrococcal nuclease fusion proteins by cultures of the double protease-minus strain B. subtilis BD170 AP^-/NP^-. Cells were grown in Veal/Yeast (VY) medium containing chloramphenicol (5 ug/ml) at 37°C with vigorous aeration. Protease inhibitors were added when the cultures reached 220 Klett units and again at the time of harvest. Cells were harvested by centrifugation, washed with 10 mM Tris, pH 8.8, 30 mM in NaCl and sonicated for 2 minutes at 0°C. Samples were taken at 300 (1,4), 370 (2,5), and 420 (3,6) Klett units. Cultures were grown in the presence of (a) 1.0 mM PMSF, (b) 1.0 mM PMSF, antipain (1.0 mg/ml), and leupeptin (1.0 mg/ml). Samples were subject to SDS-PAGE and Western analysis. Blots were incubated with rabbit antibodies to micrococcal nuclease (top) or rabbit antibodies to interleukin 1 (bottom) and detected with goat anti-rabbit IgG-alkaline phosphatase conjugate. The interleukin 1 standard was run in lane X.

Identification of the Residual Protease Genes

The N-terminal amino acid sequence of the purified RSP isolated above was determined by Edman degradation with an automated gas-phase protein sequencer (Applied Biosystems). The protein purified by ion-exchange HPLC was desalted by reverse phase chromatography on C_3 columns eluted with a linear gradient of 0-75% (v/v) acetonitrile in 0.1% trifluoroacetic acid (TFA). Autocatalytic degradation of RSP and RCP was prevented by inactivation of the enzymes by reaction with PMSF and pHMB, respectively. Amino acid analysis was performed on acid hydrolysates of the purified RSP protein. N-terminal amino acid sequencing was performed on the intact protein RSP.

As a result, the N-terminal amino acid sequence of RSP was determined to be as shown in Fig. 6. Based

on this amino acid sequence data, regions of least ambiguous DNA sequence, as predicted from code degeneracy, were identified and oligonucleotide probes corresponding to these sequences were synthesized for use in hybridization studies to locate the residual serine protease gene. Where necessary, code degeneracy was accommodated by synthesis of mixed base probes.

Suitable oligonucleotide probes are shown in Fig. 7. Of course, other probe sequences based upon other parts of the protease amino acid sequence could also be effectively used.

For example, peptide fragments of the respective proteins are separated especially by reverse phase chromatography on C_{18} columns eluted with similar solvents (i.e. acetonitrile or iso-propanol in 0.1% TFA). Appropriate fragmentations of the proteins to component peptides are made by CNBr cleavage. Fragmentation is most readily accomplished enzymically by digestions with trypsin after Lys and Arg residues, or with clostripain after Arg residues. Clostripain digestion is particularly appropriate for RSP since it has only 4 Arg residues per molecule. Other known specific cleavage methods may also be useful for generating specific peptide fragments of RSP and RCP.

Restriction digests of B. subtilis IS75 or BD170 chromosomal DNA can be prepared using HindIII and Sau3A1 restriction nucleases. Appropriately sized (approximately 3kb) RCP and RSP specific fragments resulting from the several different digests can be isolated from preparative agarose gels or from sucrose density gradients (5-20%) after non-equilibrium centrifugation at neutral pH. Plasmid libraries can be constructed that contain the isolated RSP and RCP specific fragments using pUC or pBR322 plasmid vectors cut at HindIII or Bam HI sites and cloning in E. coli. These libraries are screened for RSP or RCP specific inserts by colony hybridization with the ^{32}P-labelled

oligonucleotide probes. Plasmid DNA is prepared from positive clones and characterized by restriction analysis. For example, these restriction digests can be examined by Southern analysis using the ^{32}P-labelled oligonucleotide probes for RSP and RCP specific sequences. Fragments hybridizing with the ^{32}P-labelled probes are located by auto-radiography. With protease-specific restriction fragments in hand, inactivation of their respective protease genes can be accomplished by homologous recombination.

Creation of Site Specific Mutants

To facilitate selection of protease deficient deletion mutants, an antibiotic resistance marker can be advantageously inserted into the deleted gene in the plasmid. For example, genes encoding kanamycin, erythromycin, fusidic acid, and chloramphenicol resistance can be inserted into the deleted genes for AP, NP, RSP and RCP, respectively. The vector encoding for the mutated protease sequence and the respective antibiotic resistance marker are then used to transform B. subtilis.

Since pUC and pBR322 based plasmids cannot replicate in Bacillus, AP^-, NP^-, RSP^- and RCP^- mutants in which the deletion is incorporated into the bacterial chromosome by homologous recombination can be selected for by growth on an appropriate antibiotic-containing medium after transformation of Bacillus. Depending on the recipient host's phenotype, mutant strains multiply protease deficient, e.g. triple mutant strains having the phenotype $AP^-/NP^-/RSP^-$ and $AP^-/NP^-/RCP^-$ and quadruple mutant strains having the phenotype $AP^-/NP^-/RSP^-/RCP^-$, can be selected. For example, EcoRI-digested B. subtilis IS75 or BD170 chromosomal DNA cloned in E. coli after ligation into EcoRI-cut pUC9 vector and selection for transformants by growth in the presence of ampicillin, permits the isolation of clones encoding the genes for alkaline protease and neutral protease, pAP41 and

pNP110.2, respectively. Subcloning of the cloned DNA into M13 and sequence analysis by the dideoxy method of Sanger confirmed that these clones encode for the respective proteases. Stahl, M.L. and Ferrari, E. (1984) J. Bacteriol, 158, 411-418; Yang et al., (1984) J. Bacteriol, 160, 15-21. An internal deletion was made in both genes, by removal of a unique restriction fragment in the case of the serine protease gene, and by Bal31 deletion from a unique restriction site in the neutral protease gene. The genes encoding for erythromycin resistance and kanamycin resistance were cloned into the internal deletions created in the alkaline protease and neutral protease genes, respectively. Transformation of the constructs into B. subtilis successfully generated mutants which were defective in the alkaline protease, the neutral protease, or both alkaline and neutral proteases in any desirable B. subtilis host by homologous recombination (Fig. 1).

Removal of the Antibiotic Resistance Markers

A limitation of this approach to systematically inactivate the protease genes is that the antibiotic resistance markers remain in the host genome. In the case of $AP^-/NP^-/RCP^-/RSP^-$ mutants this entails the use of four separate antibiotic resistance markers. In turn, because of this limiting antibiotic resistance of the host, the range of useful cloning vectors is restricted to those that encode additional antibiotic resistance. Thus, ideally the resistance markers in the genome should be inactivated. Several approaches can be used to achieve this goal.

The inactivation of the protease genes could be achieved by congression and thus avoid the insertion of the antibiotic resistance marker. Lysostaphin activity can be detected by the appearance of clear halos in an agar overlay containing a suspension of live or heat killed S. aureus. Thus, inactivation of lysostaphin and the activation of prolysostaphin to

lysostaphin can be visualized in order to screen colonies to specifically detect RSP and RCP, or their inactivation, by the appearance or lack of lysostaphin-dependent halos, respectively. However, this would entail extensive screening for specific protease inactivation.

An alternative approach is the use of a transposon, for example Tn917 encoding erythromycin resistance, to disrupt the protease genes as described above. Tn917 can be ligated into the disrupted protease gene in pUC8, and used to inactive the host protease genes by homologous recombination. Recombinants can be selected by growth in the presence of erythromycin. This approach has the advantage that the transposable element can be lost from the genome when grown subsequently in the absence of erythromycin selection. This can be confirmed by sensitivity to erythromycin.

Another approach is to ligate an intact fusidic acid resistance marker to a fragment carrying a truncated chloramphenicol resistance marker (encoding the first 2/3 of the chloramphenicol acetyl transferase (cat) gene). The hybrid construct encoding fusidic acid resistance would be inserted into the deleted protease gene in the pUC vector. This construct would be used as described above to insertionally inactivate after homologous recombination the respective protease genes for RSP and RCP by selection for fusidic acid resistance.

The fusidic acid marker can likewise be inactivated by homologous recombination. The erythromycin resistant plasmid pRN5101 encodes a temperature sensitive replicon that is suitable for this purpose. A truncated fusidic acid marker (comprised of the first 1/3 of the gene) would be ligated to the latter 2/3 of the cat gene and this construct inserted into pRN5101. Transformants of the fusidic acid resistant strain would first be selected

by growth in the presence of erythromycin. Recombinants which rescue the chloramphenicol marker into the plasmid result from homologous recombinations within the fusidic acid marker (thereby inactivating this gene) and cat gene sequences. Recombination within the cat gene sequences can result in an active chloramphenicol marker residing in the host genome; this will not inactivate the fusidic acid marker. Both would be selected by growth in the presence of chloramphenicol. Growth of the recombinants at the non-permissive temperature in the absence of chloramphenicol would cure the strain of the plasmid, i.e. eliminate the plasmid form the bacterial strain. The cured strains would then be screened for sensitivity to fusidic acid to select for the appropriate recombinant.

Intellectual Property Rights in Biotechnol gy Worldwide

F. PCT FORM PCT/RO/134

Indication with Respect to Deposited Microorganisms

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 8, line 9-14 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 PARKLAWN AVENUE
ROCKVILLE, MARYLAND

Date of deposit	Accession Number
APRIL 15, 1986	67080

B. ADDITIONAL INDICATIONS (Leave blank if not applicable). This information is continued on a separate attached sheet ☐

deposited organism, designated Bacillus sphaericus 00, is deficient in four identified protease activities.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (Leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was (Authorized Officer)

Form PCT/RO/134 (January 1981)

Claims

1. A substantially pure culture of a protease-deficient gram positive bacterium substantially free of neutral protease, alkaline protease and at least one further residual protease selected from the group consisting of residual serine protease and residual cysteine protease.

2. A culture according to claim 1 wherein the bacterium is the result of mutations in the DNA sequence of the bacteria.

3. A culture according to claim 2, wherein at least one of the mutations is a deletion mutation.

4. A culture according to claim 1, wherein the bacteria is of the genus Bacillus.

5. A culture according to claim 4, wherein the bacteria is of the species Bacillus subtilis.

6. A culture according to claim 4, wherein the bacteria is of the species Bacillus sphaericus.

7. A culture according to claim 6, wherein the bacteria is Bacillus sphaericus 00.

8. A culture according to claim 3, wherein there is a deletion mutation in the gene encoding for residual serine protease.

9. An oligonucleotide probe that is substantially complementary to a part of the nucleic acid sequence encoding the 40 N-terminal amino acids of residual serine protease.

10. A probe according to claim 9, selected from the group consisting of

ATX GGN AAY GAY GT;

GAY GCN AAY GCN TTZ AA; and

GGN GAY TTZ ATX GAZ GT

wherein N is G, T, A, or C, X is T, A, or C, Y is G or A and Z is T or C.

11. A method of assaying for residual serine protease or residual cysteine protease activity in a sample comprising:

(a) mixing the sample with a solution of a suitable heterologous protein;

(b) incubating the mixture for a period of time sufficient to allow any residual serine protease or residual cysteine protease to inactivate or degrade at least a part of the heterologous protein; and

(c) measuring the degradation of the heterologous protein or the accumulation of the degradation product.

12. A method of assaying for residual serine protease activity in a sample comprising

(a) mixing the sample with a solution of lysostaphin;

(b) incubating the mixture for a period of time sufficient to allow any residual serine protease to inactivate or degrade at least a part of the lysostaphin;

(c) adding a cell suspension of Staphylococcus aureus to the incubated mixture; and

(d) comparing the number of whole Staphylococcus aureus cells in the staphylococcus-containing mixture with the number of cells in a control to which no sample was added.

13. A method of assaying for residual cysteine protease activity in a sample comprising

(a) mixing the sample with a solution of prolysostaphin;

(b) incubating the mixture for a period of time sufficient to allow any residual cysteine protease to convert at least a part of the prolysostaphin to lysostaphin;

(c) adding a cell suspension of Staphylococcus aureus to the incubated mixture; and

(d) comparing the number of whole Staphylococcus aureus cells in the staphylococcus-containing mixture with the number of cells in a control to which no sample was added.

14. A substantially purified preparation of a serine dependent protease having the N-terminal amino acid sequence depicted in Figure 6.

15. A protease according to claim 13 which has been isolated from Bacillus subtilis.

16. A substantially purified preparation of a cysteine dependent protease isolated from Bacillus subtilis, said protease being inactivated by p-hydroxymercuribenzoate and able to convert prolysostaphin to lysostaphin.

17. A method of inhibiting or inactivating the effects of residual serine protease (RSP) and residual cysteine (RCP) protease produced in a bacterial culture comprising adding to the culture an amount of a compound sufficient to inhibit or inactivate the effect of RSP and RCP.

Fig 1

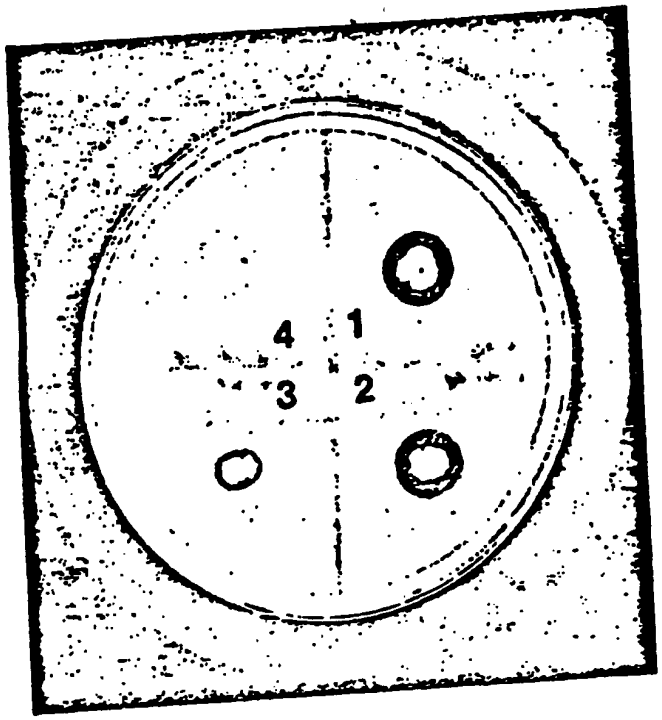


Fig 2

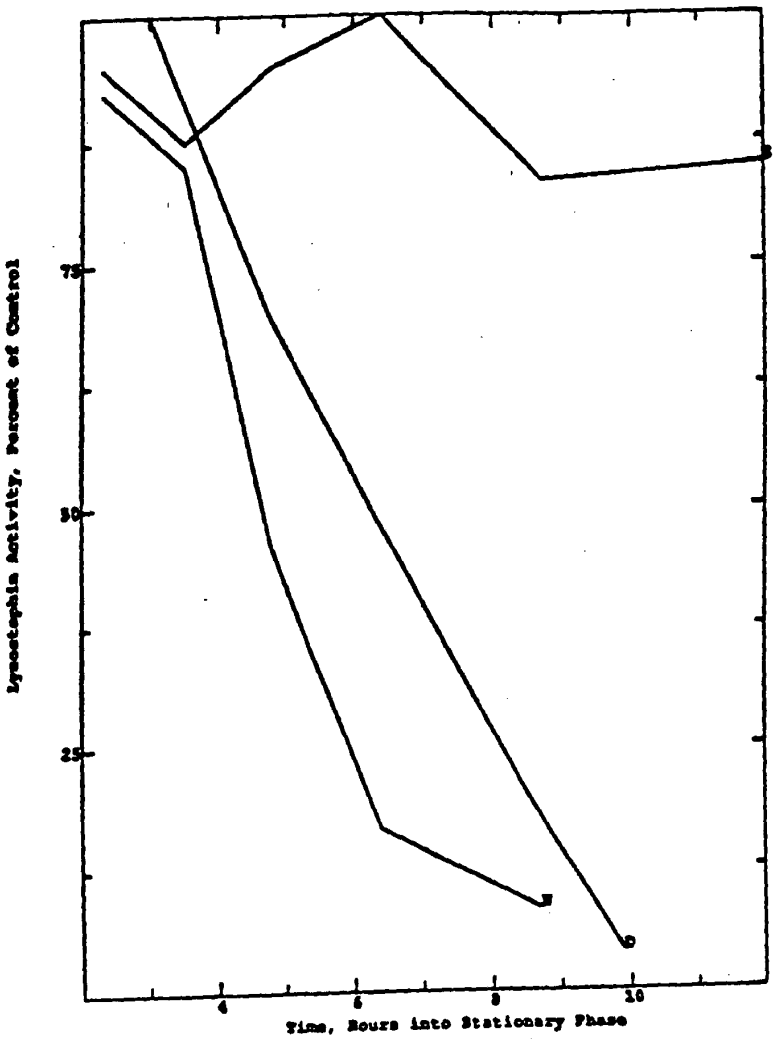
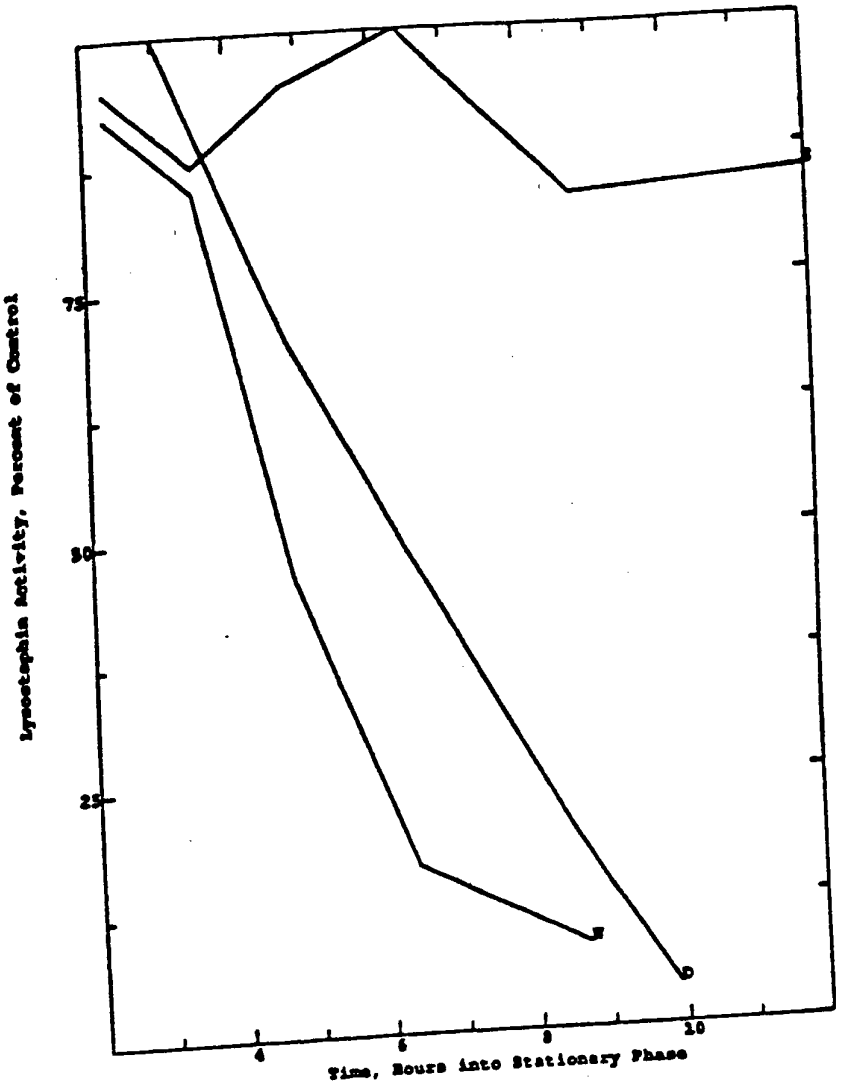
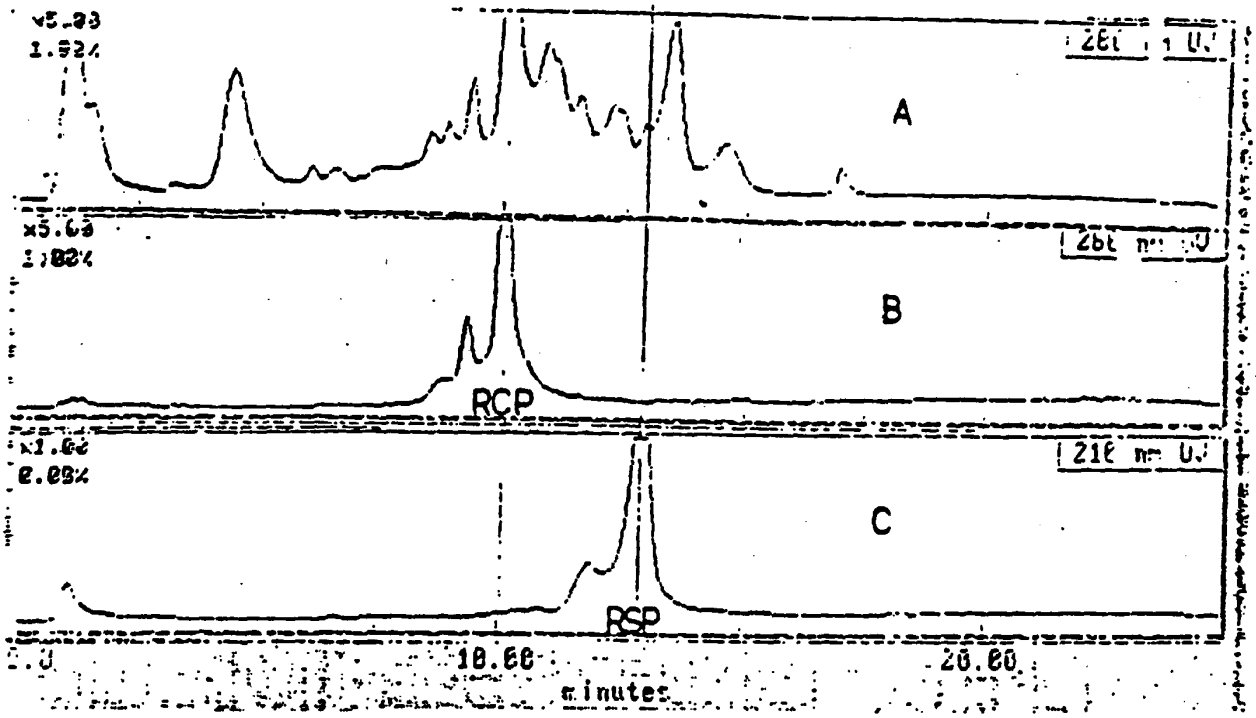
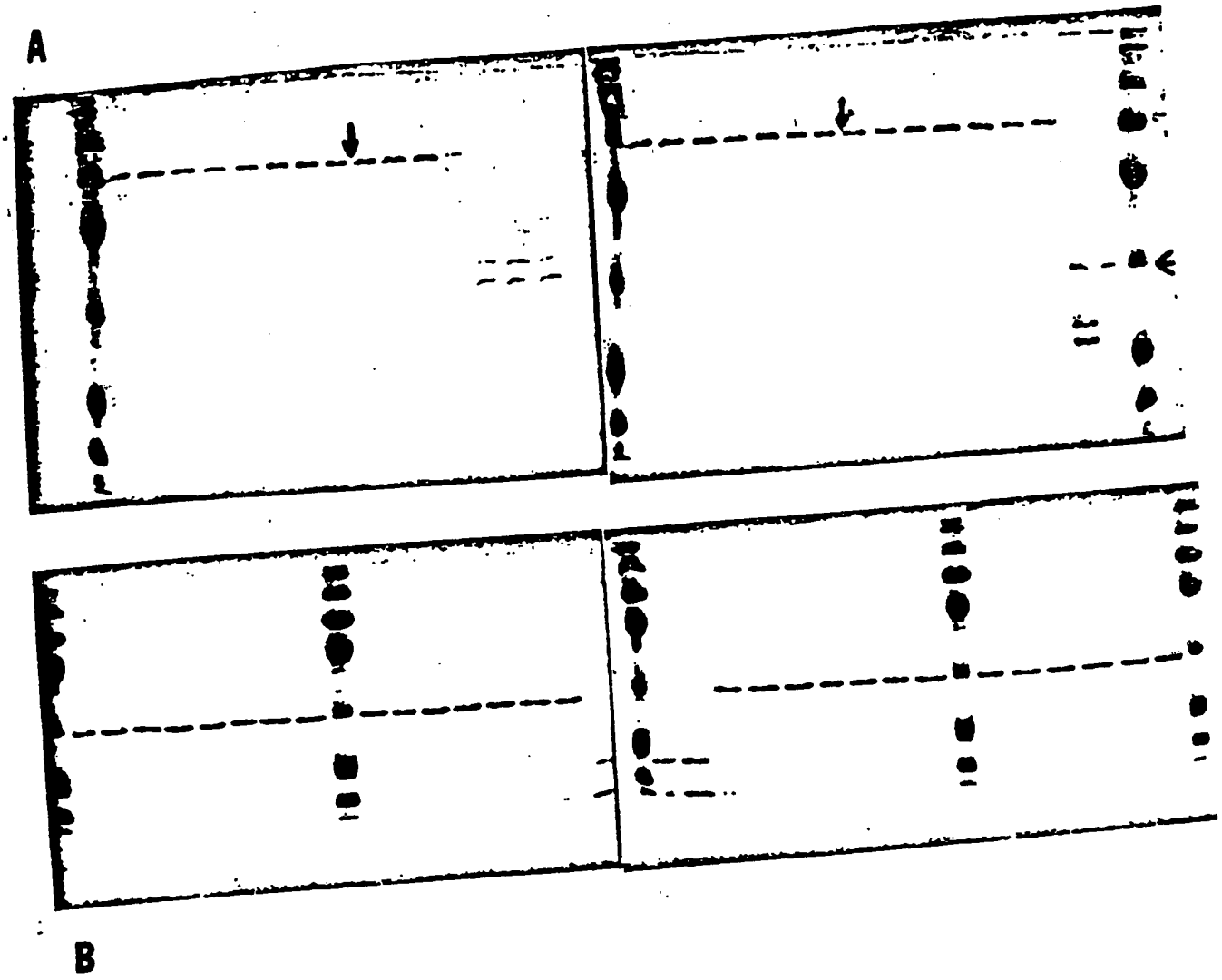

Lysostaphin Activity, Percent of Control
75
50
25
4
6
8
10
Time, Hours into Stationary Phase

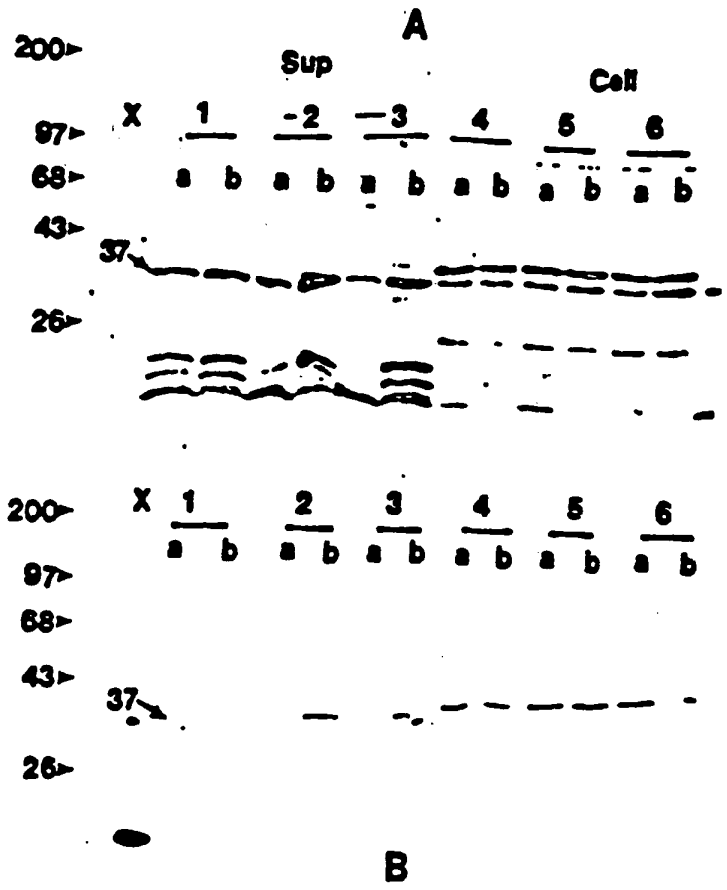
Fig 2


Lysostaphin Activity, Percent of Control
75
50
25
4
6
8
10
Time, Hours into Stationary Phase
D


A
B
RCP
C
RSP
10.00
minutes
20.00


A
B

5/6 Fig 5


A
200
97
68
43
37
26
Sup
Cell
X 1 -2 -3 4 5 6
a b a b a b a b a b a b
B
200
97
68
43
37
26
X 1 2 3 4 5 6
a b a b a b a b a b a b

Figur — 6

Amino Terminal Sequence of RSP

Leu Ile Gly Lys Glu Val Leu Pro Phe Glu Ala Lys Ala Phe Lys Asn
1 2

Gly Glu Phe Ile Asp Val Thr Asn Glu Asp Leu Lys Gly Gln Trp Ser
3

Ser Val Phe Phe Phe Tyr Pro Ala

Fig 7

Mixed DNA Probes for RSP Cloning

1. ATT GGN AAG GAG GT
   A A A
   C

2. GAG GCN AAG GCN TTT AA
   A A C

3. GGN GAG TTT ATT GAT GT
   A C A C
   C

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/01056

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12Q 1/38, C12R 1:07, C12N 15/00, C12N 9/56, C07H 21/04

US: 435/23,172.1, 252.5, 222; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6,23,172.1,184,219,222,252.1,252.5,221 536/27 935/38,47,74,78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computer Search: Dialog (CA Search,Scisearch, Biosis Previews Embase, Life Sciences Collection), Intelli Genetics (PIR,Swiss Prot,EMBL)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Journal of Bacteriology, Volume 160, No.1, 15-21, issued October 1984, M.Y. Yang et al, "Cloning of The Neutral Protease Gene of Bacillus subtilis and The Use of The Cloned Gene To Create An In Vitro-Derived Deletion Mutation", see page 16, column 2; page 17, column 1; and page 18, column 1.	1-10
Y	Abstracts of The Annual Meeting of The American Society For Microbiology, issued 1987, L.S. Heath et al, "Expression In Escherichia coli of The Gene Encoding Lysostaphin Endopeptidase", see the abstract No. H-58.	11-13
Y	Journal of Bacteriology, Volume 158, no.1, 411-418, issued May 1984, M.L. Stahl et al,	17

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 August 1989	04 OCT 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	Esther M. Kepplinger

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	"Replacement of The Bacillus subtilis Subtilisin Structural Gene With An In Vitro-Derived Deletion Mutation", see page 412, column 2 and page 415, column 1.	
Y	Journal of Bacteriology, Volume 155, No. 1, 145-152, issued July 1983, C.A. Roitsch et al, "Bacillopeptidase F: Two Forms of a Glycoprotein Serine Protease From Bacillus subtilis 168", see page 148 column 1 and page 149, column 1.	11,17
Y	Nucleic Acids Research, Volume 15, No.2, 853, issued 26 January 1987, T. Sekiguchi et al, "The Nucleotide Sequence of 3-Isopropylmalate Dehydrogenase Gene From Bacillus caldotenax", see DNA sequence, nucleotides 39-53.	9,10
Y	Gene, Volume 34, 169-177, issued 1984, D. J. Henner et al, "Nucleotide Sequence of The Bacillus subtilis Tryptophan Operon", see Figure 2.	9,10
Y	Proceedings of The National Academy of Science, USA, Volume 83, issued August 1986, C. Binnie et al, "Gene Encoding The 37 Species of RNA Polymerase Factor From Bacillus subtilis", see Figure 3, DNA encoding amino acids 139-144.	9,10
Y	J. R. Whitaker, "Principles of Enzymology For The Food Sciences", published 1972, by Marcel Dekker, Inc. (New York), see pages 515 and 526.	17